The role of intron 1 in *MDR1* regulation in drug resistant cancer cells

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Certificate of Authorship/originality

I certify that the work in this thesis has not previously been admitted for a degree nor has it been submitted as part of the requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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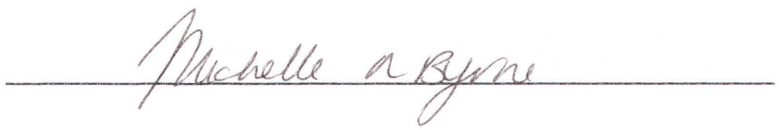

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Lastly but perhaps most importantly I would like to dedicate to this work to my grandfather and possibly biggest supporter, the late Leslie Charles Way. You are forever in my heart.

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Abbreviations

A	adenine or adenosine
ABC	ATP-binding cassette
AMP	adenosine monophosphate
ATCC	American type culture collection
ATP	adenosine triphosphate
5'-azadC	5'-azadeoxycytidine
bp	base pairs
APS	Ammonium persulphate
BSA	Bovine serum albumin
C	cytosine or cytidine
CAT	chloramphenicol acetyl transferase
cAMP	cyclic AMP
cDNA	complementary DNA
CMC	cytotoxic membrane complex
CMV	Human Cytomegalovirus
Ci	curie
CO_2	Carbon dioxide
CpG	CG dinucleotide
CRE	cAMP regulatort elements
CREB	cAMP-responsive element binding protein
CRS	cAMP response sequences
CTP	cytosine triphosphate
DEPC	diethylpyrocarbonate
DMRIE-C	1,2-dimyristyloxypropy 1-3-dimethyl-hydroxy-ethylammonunbromide
DMSO	dimethylsulphoxide
DNA	deoxribonucleic acid
DNase	deoxyribonuclease
dNTP	(unspecified) deoxyribonucleotide triphosphate
dsDNA	double stranded DNA

DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
Egr	early growth response
EGTA	ethylene glycol-bis-(β-aminoethyl ether) N, N, N', N'-tetracetic acid
EMSA	electrphoretic mobility shift assay
ET-743	Ecteinascidin-743
EtBr	ethidium bromide
5-FU	5-Fluoro-deoxyuridine
FCS	Fetal calf serum
fmole	femptomole
g	grams
G	guanine or guanosine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione
GST	glutathione S-transferase
GTP	guanine triphosphate
HAT	Histone acetylase
HDAC	Histone deacetylases
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
HSE	heat shock response element
HSF	Heat shock factor
IC_{50}	50% inhibitory concentration
INR	Initiator element
kb	kilobase
kDa	kilodalton
L	Litre
LB Medium	Luria Bertani medium
LRP	Lung resistance protein
MAC	Membrane attack complex
MCS	Multi cloning site
MDR	Multidrug resistance
MDR1	Multidrug resistance gene 1

MDR2	Multidrug resistance gene 2
mM	Millimolar
J/m^2	joules per metre squared
μM	micromolar
LMPCR	Ligation mediated PCR
ml	millilitre
mRNA	messenger RNA
MRP	multidrug resistance associated protein
MRP1	multidrug resistance associated protein gene 1
MRP2	Mulidrug resistance associated protein gene 2
MTT	3-4,5-dimethylthiazole-2,5 diphenyl tetra bromide
MW	molecular weight
nm	nanometer
nM	nanomolar
NRE	negative response element
OD	Optical density
ONPG	O-Nitrophenyl β-$_D$-Galactopyranaside
PBS	Phosphate-buffered saline
P/CAF	CREB binding protein associated factor
PCR	polymerase chain reaction
P-gp	p-glycoprotein
PKA	type 1 cAMP-dependant kinase
PKC	protein kinase C
pmole	picomole
PABP	poly(A)-binding protein
poly(A)	polyadenylated
RB	retinoblastoma
rGTP	Riboguanine triphosphate
RHA	RNA helicase A
RNA	ribonucleic acid
RNase	Ribonuclease
RT	room temperature

RT-PCR	reverse-transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SRF	Serum response factor
SSRE	Serum starvation response element
SXR	Steriod xenobiotic receptor
ssYB-1	single stranded YB-1
T	thymine or thymidine
TAE	Tris-acetate/EDTA electrophoresis buffer
TBE	Tris-borate/EDTA electrophoresis buffer
TE	Tris-EDTA
TEMED	Tetramethyl ethylene diamine
TBP	TATA binding protein
TDPCR	Terminal deoxynucleotidyl transferase PCR
TdT	Terminal dexoynucleotidyl transferase
TM	Transmembrane
TPA	12-O-tetradecanoylphorbol-13-acetate
Tris	tris(hydroxymethyl) aminomethane
TSA	trichostatin A
TSP	transcription start point
tRNA	transfer RNA
TTP	thymidine triphosphate
U	unit
UV	ultraviolet

Composition of general solutions

LB Agar

1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% Agar, pH 7.0

LB Medium

1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0

PBS

137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_47H_2O$, 1.47mM KH_2PO_4, pH 7.2

TAE (1X)

40mM Tris-acetate, ImM EDTA, pH 8.0

TBE(1X)

90mM Tris-borate/1mM EDTA

Long ranger TBE buffer (10X)

1.3M tris base/0.5M boric acid/25mM EDTA

TE Buffer (1X)

10mM Tris-HCl, 1mM EDTA (pH 7.5 or 8.0)

Loading buffer (6X)

40% sucrose, 50mM EDTA, 0.25% bromophenol blue

General reagents were prepared using deionised water and sterilized by autoclaving or filtration using a 0.22μM filter when autoclaving was inappropriate. All other solutions not specified here were also autoclaved or filter sterilized as appropriate.

Abstract

Multidrug resistance (MDR) is a major limitation in the successful treatment of cancer using chemotherapy. Cancers can be intrinsically resistant or develop resistance during treatment to a variety of structurally and functional unrelated drugs. Numerous cellular mechanisms which contribute to MDR have been identified, however, the most common mechanism is the over expression of a 170kDa glycoprotein referred to as P-glycoprotein (P-gp). P-gp acts as an ATP-dependant drug efflux pump and is encoded by the *MDR1* gene. An understanding of the regulation of the *MDR1* gene is essential if the clinical impact of MDR is to be reduced. However, at this stage how this gene is regulated *in vivo* remains elusive. Amplification of the *MDR1* gene or an increase in mRNA stability may increase expression in cells already expressing P-gp, though they do not account for activation and/or regulation of the *MDR1* gene itself. Hence the focus of many studies is on the regulation of *MDR1* transcription.

The current study investigated the role of intron 1 in *MDR1* promoter regulation. The intron 1 region has been shown to contain a CpG island which is differentially methylated in the drug sensitive HL60 cells and completely unmethylated in MDR H/E8 cells. *In vitro* DNA:protein interactions were found to exist within this differentially methylated region. Thus the aim of the current study was to determine the functional role of the downstream CpG island in *MDR1* regulation in cells of varying origin and varying MDR levels. The MDR cells lines studied were the HL60 derived H/E8 leukaemic cell line, the K562 derived Kepru leukaemic cell line, the KB-3-1 derived KB-8-5 cervical cancer cell lines and the LoVo and L/ADR colon cancer cell lines. Reporter gene studies indicated an overall negative role in reporter activity for the +283 to +606bp region in the leukaemic MDR cell lines, whilst the same region was shown to increase reporter activity in the KB-8-5 cells. The colon LoVo and L/ADR cells also showed an increase in activity for the +283 to +606bp, however, the increase was not found to be significant. Further subdivision of the +283 to +606bp region suggested the existence of several different potential and repressor and enhancer regulatory elements in the H/E8, Kepru and KB-8-5 MDR cell lines.

Similarly, reporter gene functional studies of the +65 to +282bp region resulted in an overall inhibitory effect in reporter gene activity in the H/E8 cells and a stimulatory effect in the KB-8-5, LoVo and L/ADR MDR cell lines. Further subdivision of the +65 to +282bp region revealed several different potential repressor and enhancer elements in the cell lines tested. This region had not been previously investigated for DNA:protein interactions.

UV *in vivo* footprinting was used to investigate *in vivo* chromatin structure and potential DNA:protein interactions in intron 1 of the *MDR1* gene. Sites of hypersensitivity and protection were found in intron 1 in all cell lines, however, it is hypothesised that some of the sites observed relate directly to chromatin structure as opposed to specific transcription factor DNA binding. For example a region of concentrated protection, possibly due to a positioned nucleosome was found to exist in the drug sensitive cells, whilst this same region was less and less protected in the more drug resistant cells. Furthermore, an increase in the number of hypersensitive sites was observed in the more drug resistant cell lines which may be due to RNA polymerase II pausing. However, many of the protected sites were also found throughout the intron 1 region, including some with the previously observed *in vitro* DNA footprints, providing further evidence that intron 1 is involved in the regulation of the *MDR1* gene. A comparison of the locations of the *in vivo* footprints with those of transcription factor consensus binding sites suggests many possible candidates for intron 1 mediated *MDR1* regulation that can be followed up in future studies. This study is the first to provide *in vivo* evidence for specific and structural features and possible transcription factor mediated regulation in intron 1 of the *MDR1* gene.